A deoxyribonucleotidase in mitochondria: Involvement in regulation of dNTP pools and possible link to genetic disease

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Three cytosolic and one plasma membrane-bound 5′-nucleotidases have been cloned and characterized. Their various substrate specificities suggest widely different functions in nucleotide metabolism. We now describe a 5′-nucleotidase in mitochondria. The enzyme, named dNT-2, dephosphorylates specifically the 5′- and 2′(3′)-phosphates of uracil and thymine deoxyribonucleotides. The cDNA of human dNT-2 codes for a 25.9-kDa polypeptide with a typical mitochondrial leader peptide, providing the structural basis for two-step processing during import into the mitochondrial matrix. The deduced amino acid sequence is 52% identical to that of a recently described cytosolic deoxyribonucleotidase (dNT-1). The two enzymes share many catalytic properties, but dNT-2 shows a narrower substrate specificity. Mitochondrial localization of dNT-2 was demonstrated by the mitochondrial fluorescence of 293 cells expressing a dNT-2-green fluorescent protein (GFP) fusion protein. 293 cells expressing fusion proteins without leader peptide or with dNT-1 showed a cytosolic fluorescence. During *in vitro* import into mitochondria, the preprotein lost the leader peptide. We suggest that dNT-2 protects mitochondrial DNA replication from overproduction of dTTP, in particular in resting cells. Mitochondrial toxicity of dTTP can be inferred from a severe inborn error of metabolism in which the loss of thymidine phosphorylase led to dTTP accumulation and aberrant mitochondrial DNA replication. We localized the gene for dNT-2 on chromosome 17p11.2 in the Smith–Magenis syndrome-critical region, raising the possibility that dNT-2 is involved in the etiology of this genetic disease.

Mitochondrial DNA synthesis occurs throughout the whole cell cycle, independent of nuclear DNA replication (1). It is catalyzed by a separate DNA polymerase that uses distinct 2′-deoxyribonucleoside 5′-triphosphate (dNTP) pools (2, 3), sequestered from cytosolic dNTPs by the mitochondrial membranes. What is the origin of mitochondrial dNTPs and how are pool sizes regulated? Despite considerable efforts, a mitochondrial ribonucleotide reductase has not been found, suggesting import of dNTPs or deoxyribonucleosides from the cytosol into mitochondria. dNTPs are synthesized by the cytosolic ribonucleotide reductase and can be imported directly by a permease of the mitochondrial membrane (4). Deoxyribonucleosides are derived from the extracellular fluid or by catabolism of dNTPs and, after import into mitochondria, phosphorylated by specific intramitochondrial deoxyribonucleoside kinases (5–8).

But what regulates the size of intramitochondrial dNTP pools? This is an important question, because it is well established for cytosolic dNTPs that pool imbalance is genotoxic (9) and can cause specific diseases. Thus, in some cases of hereditary severe immune deficiency, the accumulation of dATP (10, 11) or dGTP (12, 13) in the cytosol of blood cells results in the apoptotic destruction of B and/or T cells. In a different autosomal recessive disease, neurogastrointestinal encephalomyopathy, dTTP accumulates (14) and results in defects in mitochondrial DNA replication. This disease demonstrates that intramitochondrial dNTP pools also must be regulated. In all three diseases, dNTP accumulation is the result of genetic defects in enzymes involved in the catabolism of deoxyribonucleosides: adenosine deaminase (10) or purine nucleoside phosphorylase (12) for purine dNTPs, and thymidine phosphorylase for dTTP (14).

Cytosolic dNTP pools are regulated by two different mechanisms: (*i*) by allosteric regulation of their *de novo* synthesis by ribonucleotide reductase (15); and (*ii*) by substrate cycles, involving the dynamic interplay between anabolic deoxyribonucleoside kinases and catabolic deoxyribonucleotidases (16). The second mechanism helps to explain why the loss of a catabolic enzyme can result in the accumulation of a specific dNTP.

In substrate cycles, a kinase and a 5′-nucleotidase catalyze opposing irreversible reactions (Fig. 1). When the supply of deoxyribonucleotides exceeds the requirements for DNA replication, the surplus is degraded and leaves the cell, whose membrane is permeable for deoxyribonucleosides, but not deoxyribonucleotides. When dNTPs are in short supply, deoxyribonucleosides are imported into cells, phosphorylated, and used for DNA replication. Other enzymes that degrade deoxyribonucleosides, such as adenosine deaminase and phosphorylases, shift the cycle into the direction of catabolism. These enzymes attain special importance in cells of the lymphoid system that are low in deoxyribonucleotidase activity, and, in their absence, dATP and dGTP specifically accumulate in B and T cells and cause disease.

The nature of several kinases involved in cytosolic substrate cycles has been known for some time from work with mutant cell lines deficient in their activity (17–19). The nature of the deoxyribonucleotidase(s) is less clear. Recently we described the cloning of a mouse cDNA for a ubiquitous cytosolic deoxyribonucleotidase (20) that participates in some cytosolic deoxyribonucleoside/deoxyribonucleotide substrate cycles (C. Gazziola, V.B., and P.R., unpublished work). We now demonstrate the presence in mitochondria of a deoxyribonucleotidase with a high specificity for the 5′- and 2′(3′)-phosphates of thymidine and deoxyuridine. We suggest that this enzyme participates in a thymidine/dTMP substrate cycle and thereby regulates the size of the intramitochondrial dTTP pool. We will distinguish between the two deoxyribonucleotidases by naming the cytosolic enzyme dNT-1 and the mitochondrial enzyme dNT-2. From the location of the gene for dNT-2 on chromosome 17p11.2 in the critical region for the Smith–Magenis syndrome (21), the pos-

Abbreviations: dNT-1, cytosolic 5′(3′)-deoxyribonucleotidase; dNT-2, mitochondrial 5′(3′)-deoxyribonucleotidase; DNAm, coding sequence for complete dNT-2; DNAt, coding sequence for truncated dNT-2, lacking the mitochondrial leader sequence; GFP, green fluorescent protein; EST, expressed sequence tag; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; UTR, untranslated region.

Data deposition: The nucleotide sequences reported in this paper have been deposited in the GenBank database (accession nos. AF210652 and AJ277557).

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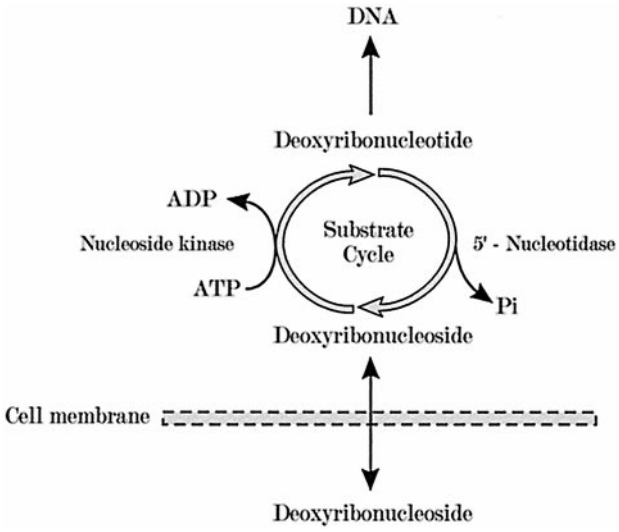

Fig. 1. Substrate cycle between a deoxyribonucleoside and deoxyribonucleotide.

sibility arises that the enzyme is involved in the etiology of this hereditary disease.

Experimental Procedures

Identification of dNT-2 cDNA and Cloning in Bacterial and Mammalian Expression Vectors. An unamplified human skeletal muscle cDNA library (Edge BioSystems, Gaithersburg, MD) was screened by PCR with forward primer NT2.2 (5′-GGTCAGAGCCCCACAGAGGAGCCCGAAG-3′) and reverse primer NT2.5 (5′-GGCCGTGGAAGCTGTCAAGGAGATGG-3′), both modeled on the genomic sequence contained in a 93.7-kb human genomic clone present in the GenBank database with accession no. AC006071. The screen produced one positive clone that contained the cDNA of dNT-2. Its coding sequence was subcloned into pET20b (Novagen) as an *Nde*I–*Bam*HI fragment obtained by PCR from the full-length cDNA using reverse primer NT2.10 (5′-CTGAGGATCCAGCCCCACAGAGGA-3′) and either NT2.9 (5′-CATACATATGATCCGGCTGGGCGG-3′) or NT2.14 (5′-CATACATATGGGAGGCCGCGCCCTACG-3′) as forward primer. The former PCR produced the complete coding sequence for dNT-2, hereafter called DNAm, the latter a sequence lacking the codons for the 31 NH_2-terminal amino acids, but including an NH_2-terminal methionine, hereafter called DNAt. The first pET construct was named p1M-dNT2 and the second p2M-dNT2.

For expression in mammalian cells, we prepared two ecdyson-inducible derivatives of pIND-GFP (22). They carried either DNAm (p1dNT2-GFP) or DNAt (p2dNT2-GFP) ligated in frame to the GFP sequence as *Hin*dIII–*Bam*HI fragments in which the stop codons had been removed by PCR. The two inserts were obtained from the complete cDNA in PCR reactions run with either NT2.16 (5′-CATCAAGCTTCTGGGCCATGATCCG-3′) or NT2.20 (5′-CATCAAGCTTGGCAGGATGGGAGGCCGCGCCCTA-3′) as forward primer and reverse primer NT2.17 (5′-CTGAGGATCCCAGCAGGGCCGCTTGC-3′).

Expression of dNT-2 in Bacteria and Enzyme Purification. The recombinant plasmids p1M-dNT2 and p2M-dNT2 were transformed into *Escherichia coli* BL21(DE3)*plysS*. The bacteria were grown at 37°C to an OD_{600} of 0.6 and induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG) for 3 h. Extracts were prepared as described (20) and used for Western blots after 12% SDS/PAGE and for the purification of dNT-2. The enzyme was purified by ammonium sulfate fractionation, chromatography with a sodium chloride gradient (from 50 to 150 mM in 30 mM Tris·HCl, pH 7.5) on DE52 cellulose (activity peak at 75 mM NaCl), followed by chromatography on Sephacryl 100 (Amersham Pharmacia) in 0.5 M KCl/30 mM Tris·HCl (pH 7.5) (protein peak at approximately 45 kDa). The protein after the DE52 step was estimated to be 60%–70% pure and gave a predominant band at ≈25 kDa on SDS/PAGE. It was used for the characterization of the catalytic properties of dNT-2.

Nucleotidase Assays. Enzyme activity was determined either with [^{3}H]dUMP as substrate or from the release of P_i from other nonlabeled nucleotides (20). Briefly, incubations were at 37°C for 30 min with 2 or 5 mM substrate, 10 mM Mg^{2+}, 30 mM KCl, and 50 mM Tris·maleate (pH 5.5), or 50 mM Tris·HCl (pH 7.5), in a final volume of 0.02 or 0.05 ml. All assays were done with at least two different concentrations of enzyme to assure proportionality.

Measured under optimal conditions at 37°C with dUMP as substrate, the enzyme had a specific activity of 150–350 units/mg protein (one unit = 1 μmol of deoxyuridine formed per minute).

Northern and Western Blots. Tissue distribution of mRNAs for dNT1 and dNT2 was determined with a human multiple tissue blot (CLONTECH). The labeled probes used for the hybridization of the mRNAs were constructed by PCR from the complete inserts of expressed sequence tag (EST) clone AA404645 (dNT-1) and EST clone AA446187 (dNT-2). A probe for actin mRNA was used for titration of mRNA loading.

Western blots of proteins extracted from overproducing bacteria were developed with affinity-purified Abs obtained from eggs of a hen immunized with a truncated form of the mouse dNT-1 (20) lacking the 10 NH_2-terminal amino acids. Alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) were used at a 1:10,000 dilution as secondary Ab.

Expression of Recombinant Deoxyribonucleotidases Fused to the GFP in Human 293 Cells. The inducible vectors coding for the deoxyribonucleotidases fused to the GFP sequence were transfected by calcium phosphate precipitation into cells of clone 293-2-100 (22*)*. This clone constitutively produces an intracellular ecdyson receptor that can be activated by the synthetic ecdyson analog ponasterone A (Invitrogen). A stable inducible line carrying p1dNT2-GFP was isolated by selection with 0.6 mg/ml G418 (GIBCO). Experiments with p2dNT2-GFP and the murine deoxyribonucleotidase linked to the GFP (expressed from vector pdNT-GFP) were done with transiently transfected cells. In all cases, cells were induced for 24 h with 4 μM ponasterone A and were examined by fluorescent confocal microscopy (excitation at 488 nm, emission at 522 nm).

Expression of dNT-2 *in Vitro* and Import into Rat Mitochondria. The full-length form of dNT-2, including the mitochondrial leader sequence and the truncated protein lacking the N-terminal 31 aa, was produced *in vitro* from p1M-dNT2 and p2M-dNT2, respectively, in a reticulocyte transcription-translation system (Promega) with ^{35}S-labeled methionine according to the instructions of the supplier. The reaction products were analyzed by 12% SDS/PAGE and detected autoradiographically. The radioactive proteins were incubated for import by liver or brain mitochondria (23), essentially according to a procedure described for yeast mitochondria (24). The radioactive proteins present in 0.003 ml of the reticulocyte system were incubated with 0.05 mg of mitochondria in 0.25 M sucrose/10 mM Hepes buffer (pH 7.5)/1 mM DTT/1 mM EDTA/20 mM succinate/5 mM methionine at 30°C for 30 min in a final volume of 0.05 ml. Freshly prepared mitochondria were used either directly or after preincubation for 5 min with 1 μM carbonylcyanide-*p*-trifluoromethoxyphenyl

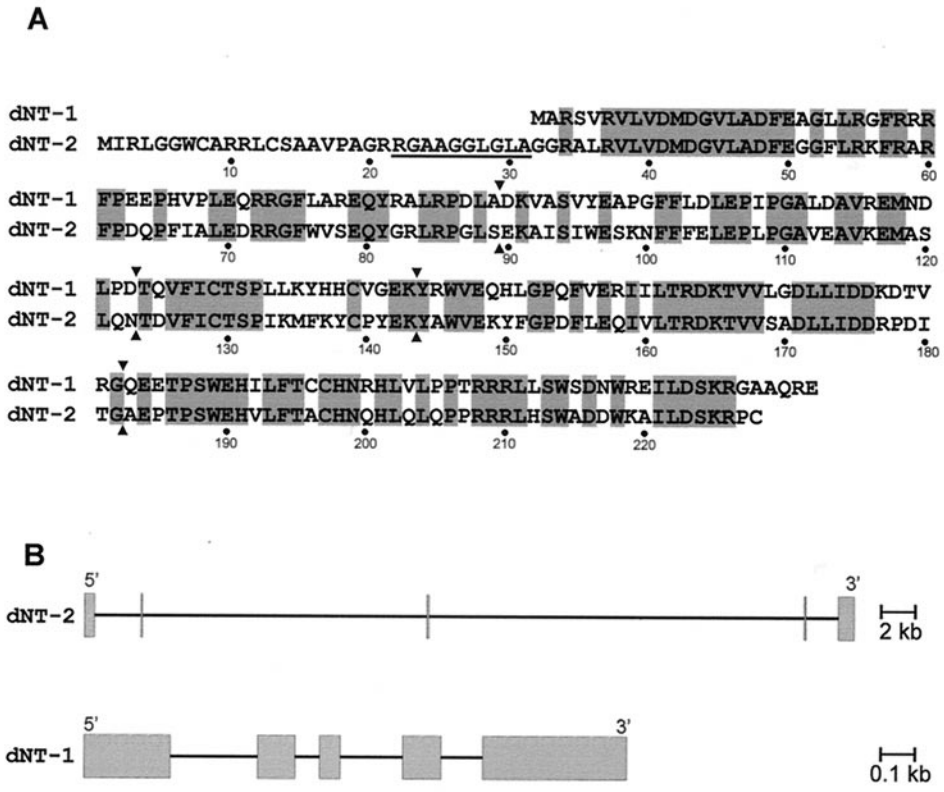

Fig. 2. Comparison of dNT-2 with dNT-1. (*A*) Amino acid sequences of the two human enzymes, deduced from their cDNA sequences. Amino acid identities are indicated by shading. The peptide motif (26) for mitochondrial two-step processing is underlined. The common exon/intron borders are indicated by arrow heads. (*B*) Organization of the genes for the two enzymes. Exons are shaded. Notice the difference in scales.

hydrazone (FCCP). The contents of the tubes were divided into two halves. One-half was centrifuged directly; the other half was treated in an ice bath for 10 min with 1 mg/ml proteinase K, followed by an additional 10 min with 1 mM PMSF and centrifugation. In both cases, the pelleted mitochondria were analyzed by SDS/PAGE, and the radioactive bands were visualized with a Packard Cyclone Phosphor Imaging System.

Results

Evidence for a second deoxyribonucleotidase (dNT-2) came from our finding of human and mouse ESTs, as well as human genomic sequences, homologous to but different from the cDNA sequence of dNT-1. From this information we could isolate, by PCR technology, the complete cDNA for dNT-2 from a human muscle cDNA library. The full-length cDNA sequence is 1617 bp long. It contains a short upstream ORF spanning nucleotides 146–220 and includes a 216-bp 5′-untranslated region (UTR) and a 714-bp long 3′-UTR starting at nucleotide 904. The cDNA translates into the amino acid sequence shown in Fig. 2*A*, where it is aligned with the sequence of the human dNT-1. Our earlier reported human dNT-1 sequence (20) lacked the 49 NH_2-terminal amino acids, present in a more recent EST clone (GenBank accession no. AK000419). Excluding the 32 NH_2-terminal amino acids of dNT-2, the two sequences are 52% identical throughout the whole length of the proteins. The unique NH_2 terminus of dNT-2 has the hallmarks of a mitochondrial leader sequence (25): a potential α-helix structure and a predicted isoelectric point of 11.8 (4 arginines and no acidic residues); furthermore the sequence RG($\downarrow$)AAGGLGLA($\downarrow$) starting at position 22 provides the required structural basis for two-step processing for import into the mitochondrial matrix. The two arrows indicate the presumed sites for processing. Typically, an arginine is located at position -10 (relative to the NH_2 terminus of the mature protein), followed by F/L/I at position -8 and T/S/G at position -5 (26). The presence of A in position -8 in our enzyme, instead of F/L/I, was recently also found in the mitochondrial forms of thioredoxin (27) and thioredoxin reductase (28). The primary structure of dNT-2 thus suggests that the protein is a deoxyribonucleotidase, similar to the cytosolic enzyme (20), but that it has a mitochondrial localization.

Based on this cDNA sequence, we identified the structure of the gene for the protein within a genomic clone from chromosome 17 (accession no. AC006071). Compared with the genomic sequence, the cDNA sequenced by us differed at two sites: we found a T instead of a C at position 71 in the 5′-UTR and a G instead of a C at position 1084 in the 3′-UTR. Nucleotide 1 of the cDNA corresponds to nucleotide 35,649 in the genomic clone. The gene covers 44 kb and consists of 5 exons and 4 introns. It lies $\approx$22 kb downstream from nucleotide 1 of the sequence coding for subunit 3 of Cop9 (29), which runs in the opposite orientation. The clone contains the initial 13.5 kb of the genomic sequence for subunit 3. The close proximity to the gene for subunit 3 of Cop9 allowed us to locate the dNT-2 gene in the critical region for the Smith–Magenis syndrome at 17p11.2 (29, 30).

In a different genomic sequence from chromosome 17 (accession no. AC022211), we could identify the structure of the gene for dNT-1 and compare the two genes (Fig. 2*B*). They show the same organization, with exon/intron borders located at identical exon sites, indicated by arrowheads in Fig. 2*A*. The dNT-1 introns are much shorter and the dNT-1 gene, whose precise location on chromosome 17 is not known, is only 1.55 kb long.

Northern blots using filters with mRNAs from various human organs suggested that the genes for the two deoxyribonucleoti-

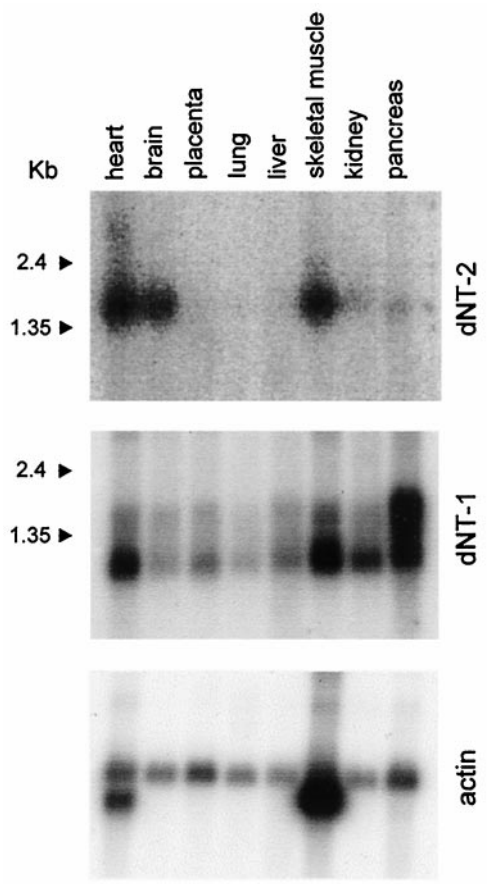

Fig. 3. Northern blots of mRNAs for human dNT-1 and dNT-2. A corresponding blot for actin-mRNA is shown for titration of mRNA loading.

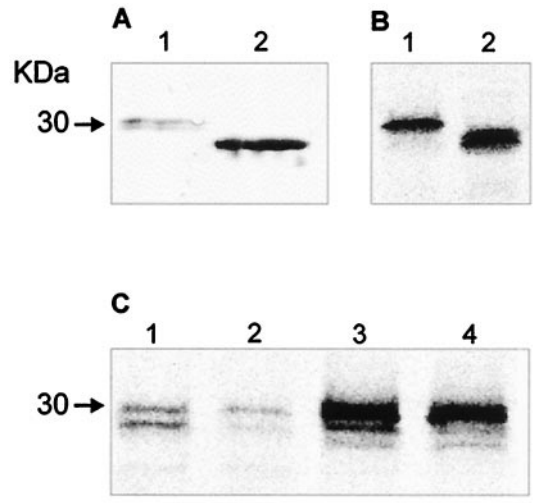

Fig. 4. Evidence for the mitochondrial localization of dNT-2. (*A*) Western blots of extracts from bacterial overproducers. Lane 1 shows the signal with a polyclonal chicken Ab against dNT-1 of an extract from *E. coli* transformed with DNAm; in lane 2, the bacteria had been transformed with DNAt. (*B*) Autoradiogram of proteins produced in a reticulocyte transcription—translation system (Promega) from DNAm (lane 1) or DNAt (lane 2). (*C*) Import of radioactive DNAm-coded protein by rat brain mitochondria. The radioactive protein from lane 1 in *B* was incubated with brain mitochondria and treated as described under *Experimental Procedures*. In the experiment of lanes 2 and 4, the mitochondria were pretreated with 1 μM FCCP to deenergize the inner mitochondrial membrane. Each incubation mixture was then divided into two equal halves: the mitochondria from one half were electrophoresed immediately on an SDS gel (lanes 3 and 4); in the second half, they were first treated with proteinase K before electrophoresis (lanes 1 and 2).

dases are expressed differently (Fig. 3). dNT-1 mRNA was found in all organs, with the highest expression in pancreas, heart, and skeletal muscle. dNT-2 mRNA was present in heart, brain, and muscle; barely visible in kidney and pancreas; and absent from placenta, liver, and lung. This type of distribution is common for mitochondrial enzymes.

To study the enzymatic activity of dNT-2, we transformed *E. coli* with either the complete coding region of its cDNA (DNAm) or with a truncated version devoid of the region coding for the 31 NH_2-terminal amino acids but with an added 5′-terminal AUG (DNAt). Extracts from *E. coli* transformed with either construct contained a highly active deoxyribonucleotidase, absent from the controls. On a Western blot developed with a chicken Ab raised against dNT-1, we found signals in the positions expected from proteins encoded by DNAm or DNAt, suggesting an immunological relation between dNT-2 and dNT-1 (Fig. 4*A*). Both constructs also served as templates in a reticulocyte transcription–translation system and produced radioactive protein bands with positions on gel electrophoresis corresponding to those on the Western blot (Fig. 4*B*). The radioactive proteins were incubated with a liver or brain mitochondrial import system (24). The mitochondria imported the DNAm protein, but not the DNAt protein. After import of the DNAm protein, two radioactive bands were found by gel electrophoresis: one corresponded to the original band, the other moved to the position of the DNAt protein (Fig. 4*C*). Protein import was blocked when FCCP was included in the system, demonstrating that the import reaction depended on energization of the mitochondrial membrane. Collectively, our data strongly suggest that DNAm codes for a deoxyribonucleotidase that can be imported by mitochondria and is truncated during import to a protein lacking the mitochondrial leader sequence.

The mitochondrial localization of the enzyme was further demonstrated in experiments in which DNAm and DNAt, after fusion of their 3′ ends to the cDNA of GFP (31), were transformed into human 293 cells. In both constructs, the DNAs were under the control of the ecdyson-inducible promoter (32). After induction, the proteins were localized in the cells from their fluorescence. The results were strikingly different for the two constructs: DNAm gave a granular fluorescence that colocalized with mitochondria visualized by staining with tetramethylrhodamine methyl ester (Fig. 5 *A–C*), whereas the fluorescence of DNAt was spread diffusely throughout the cytoplasm (Fig. 5*D*). A diffuse localization was also found in cells transfected with a GFP-coupled cDNA for dNT-1 (Fig. 5*E*).

The truncated protein was purified close to homogeneity from the *E. coli* overproducer and characterized with respect to its catalytic behavior. The enzyme had an acid pH-optimum between 5.0 and 5.5 and showed an absolute requirement for Mg^{2+}. K_M values for all substrates were between 0.1 and 0.15 mM. ATP had no effect on the reaction. The substrate specificity of dNT-2 was narrower than that of dNT-1, with enzyme activity essentially limited to the 5′- and 2′(3′)-deoxyribonucleotides of either uracil or thymine, both at the pH optimum of the enzyme and at a more physiological pH of 7.5 (Table 1). The marginal activity with dIMP and dGMP most clearly distinguishes dNT-2 from dNT-1.

To establish the presence of dNT-2 in normal mitochondria, extracts from liver, heart, and brain mitochondria from rats were assayed for 5′-nucleotidase activity. dNT-2 is recognized in crude extracts from its limited substrate specificity, its absolute dependence on Mg^{2+} and insensitivity to ATP, and its lack of dGMP and dIMP phosphatase activity. Ectonucleotidases do not

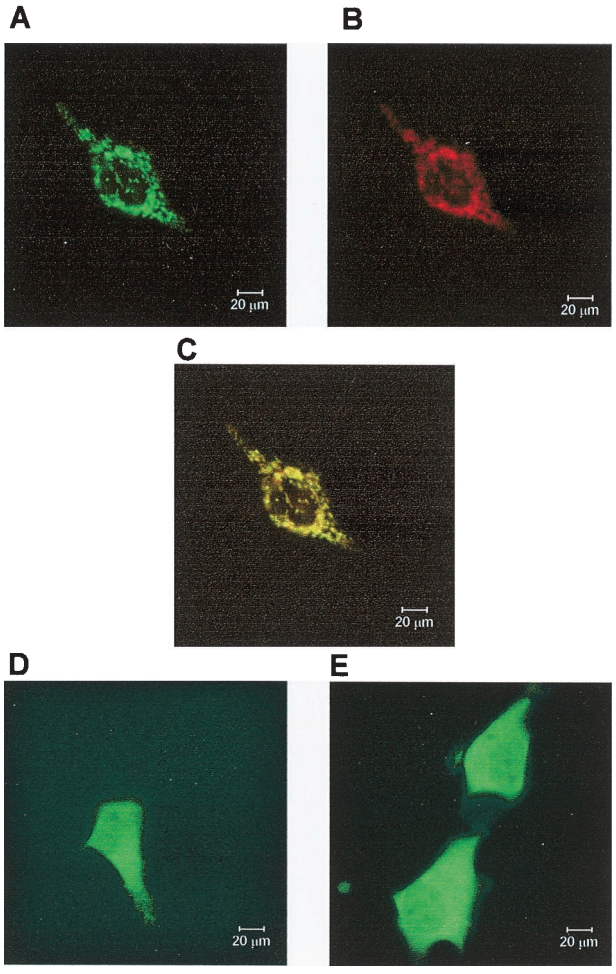

Fig. 5. Mitochondrial localization of dNT-2 shown by laser confocal microscopy. 293-2-100 cells were transfected with ecdyson-inducible vectors expressing the deoxyribonucleotidases as GFP-fusion proteins. (*A–C*) Fluorescence of the identical cell overproducing full-length dNT-2. (*A*) GFP fluorescence; (*B*) fluorescence of mitochondria stained with tetramethylrhodamine methyl ester; (*C*) overlay of *A* and *B* showing colocalization of dNT-2 and mitochondria. (*D*) GFP fluorescence of cells overexpressing dNT-2 without mitochondrial leader. (*E*) GFP fluorescence of cells overexpressing dNT-1.

absolutely require Mg^{2+} and are strongly inhibited by ATP (33). High-K_m 5′-nucleotidases are, instead, strongly stimulated by ATP (34). Mitochondrial extracts contained little dNT-1 or high-K_m 5′-nucleotidase activity. However, all extracts showed ectonucleotidase activity, with brain and heart mitochondrial extracts giving specific activities of 3 to 5 milliunits per mg of protein, and liver mitochondrial extracts corresponding values of 50 to 100. Heart and brain mitochondrial extracts showed dNT-2 activity (specific activities 5 to 10 milliunits per mg of protein). In liver preparations, the high ectonucleotidase activity, probably caused by microsomal contamination, prevented a meaningful analysis. The activity in brain mitochondrial extracts was purified 5-fold by chromatography on Sephacryl 100 and then migrated in the position expected from dNT-2 purified from the *E. coli* overproducer. These experiments clearly demonstrate the presence of dNT-2 activity in brain and heart mitochondria.

Discussion

The mitochondrial deoxyribonucleotidase described here is closely related to the previously described and cloned cytosolic dNT-1 (20). Their relatedness is evident both from the primary

Table 1. Comparison of substrate specificities of dNT-1 and dNT-2

	Relative enzyme activity, %		
	dNT-2		
Substrate	pH 5.5	pH 7.5	dNT-1
5′-dUMP	100	100	100
5′-dTMP	50	48	65
5′-dCMP	0	0	16
5′-dGMP	6	2	45
5′-dAMP	2	1	10
5′-dIMP	8	3	96
5′-UMP	30	37	16
5′-CMP	0	0	0
5′-GMP	2	0	4
5′-AMP	0	1	1
5′-IMP	2	1	11
3′-dTMP	77	103	58
3′-UMP	47	34	35
3′-CMP	0	0	0
2′-UMP	18	15	11
2′-GMP	2	0	1
dTDP	1	1	
dTTP	0	0	

Incubations were with 2 mM substrate as described in *Experimental Procedures*. With dUMP as substrate, dNT-2 had a specific activity of 340 at pH 5.5 and 220 at pH 7.5 (= 100% activity). The values for dNT-1 (100% = specific activity of 370) are taken from a previous publication (20).

structures of the two proteins and from the closely related organization of their genes, suggesting a gene duplication event. Also, their catalytic functions are similar. Both enzymes prefer 5′- and 2′(3′)-deoxyribonucleotides over 5′-ribotides as substrates. The substrate specificity of dNT-2 is, however, essentially limited to thymine and uracil deoxyribonucleotides, whereas dNT-1 also dephosphorylates purine and cytosine nucleotides. The specificity and localization of dNT-2 suggests a function of the enzyme in the regulation of the intramitochondrial dTTP pool.

dNT-2 is probably located in the inner membrane space of mitochondria. Such a location is suggested from the sequence of the leader sequence as well as from the dependence of the import of the preprotein on energization of the membrane (24–26). We found dNT-2 activity in mitochondria from both brain and heart, but not from liver. The latter result is inconclusive because the extracts contained large amounts of ectonucleotidase activity that may have masked dNT-2 activity. Also, in Northern blots, the mRNA of the enzyme was detected mainly in brain, heart, and muscle. This specificity may, at least in part, reflect the high content of mitochondria in these three organs and does not exclude the presence of the enzyme also in other organs.

The demonstration of the existence of a mitochondrial 5′-deoxyribonucleotidase leads us to two suggestions: (*i*) the enzyme probably functions in the regulation of a dTTP pool specifically used for mitochondrial DNA replication; and (*ii*) genetic loss of the enzyme may be involved in the etiology of mitochondrial diseases.

As to the regulatory function of dNT-2, we suggest that the enzyme, together with thymidine kinase 2 (5, 6), participates in a substrate cycle (cf. Fig. 1) that protects mitochondrial DNA polymerase from an excess of dTTP. Such a function appears to be particularly required for the replication of mitochondrial DNA in resting cells. In these cells, ribonucleotide reduction comes close to a standstill (35) and no longer provides dNTPs or regulates dNTP pool sizes. Cytosolic dNTP pools decrease

sharply (16). Mitochondrial dNTPs then arise from the local phosphorylation of deoxyribonucleosides, imported from the extracellular space via the cytosol. This process requires regulation. With dNT-2 in place, the stage is set for a mitochondrial thymidine/dTMP substrate cycle, similar to the corresponding substrate cycle in the cytosol (16, 18).

A possible link of dNT-2 to human disease is suggested from two circumstances. First, the enzyme functions as a barrier against the accumulation of a large dTTP pool, as does thymidine phosphorylase. The genetic loss of the latter enzyme leads to an inborn error of metabolism that specifically affects mitochondria (14). Thymidine phosphorylase is a cytosolic enzyme that degrades thymidine to thymine. In its absence, the blood of affected patients contains high levels of thymidine that can feed into the intracellular dTTP pool, overtax the thymidine/dTMP cycle and lead to aberrant mitochondrial DNA replication. It is not unlikely that a similar situation would apply to an impairment in dNT-2 activity.

A second potential link between dNT-2 and genetic disease derives from the location of its gene in the critical chromosomal region that is affected in most cases of Smith–Magenis syndrome (21, 30). This is a multiple congenital anomalies syndrome with moderate mental retardation, usually associated with a heterozygous deletion of a region on chromosome 17p11.2 that contains three repeat clusters that apparently are hot spots for homologous recombination (36). The extent of the deletion varies, but a common region with a minimal length of 1.5–2 megabases has been defined (30). The region may contain a single critical gene or several linked dosage-sensitive genes responsible for the Smith–Magenis phenotype. dNT-2 is present in this region and should now be included as a candidate gene in the etiology of the Smith–Magenis syndrome, suggesting the interesting possibility that the disease may have a mitochondrial component. To clarify the physiological function of dNT-2, it will be interesting to conduct experiments aiming at the production of genetically modified mice.

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